

POLYMORPHISM III: LINKAGE OF ATOPY TO A LOCUS ON CHROMOSOME 13

This invention is concerned with methods for the diagnosis of
5 atopic disease and with materials and methods relating thereto.

Atopy is a tendency to develop high levels of IgE and immediate hypersensitivity to allergens. Atopic diseases include hay fever, infantile eczema and most forms of asthma. Asthma is a disease which is becoming more prevalent and is the most common disease of childhood (1). Most
10 asthma in children and young adults is initiated by IgE mediated allergy (atopy) to inhaled allergens such as house dust mite and cat dander. However, not all asthmatics are atopic, and most atopic individuals do not have asthma, so that factors in addition to atopy are necessary to induce the disease (2,3). Asthma is strongly familial, and is due to the interaction
15 between genetic and environmental factors. The genetic factors are thought to be variants of normal genes ("polymorphisms") which alter their function to predispose to asthma.

Asthma may be identified by recurrent wheeze and intermittent air flow limitation. An asthmatic tendency may be quantified by the
20 measurement of bronchial hyper-responsiveness in which an individual's dose-response curve to a broncho-constrictor such as histamine or methacholine is constructed. The curve is commonly summarised by the dose which results in a 20% fall in air flow (PD20) or the slope of the curve between the initial air flow measurement and the last dose given (slope).

25 In the atopic response, IgE is produced by B-cells in response to allergen stimulation. These antibodies coat mast cells by binding to the high affinity receptor for IgE (FcεRI). When a multivalent allergen binds to an IgE-coated mast cell, the cross-linking of adjacent IgEs by allergen initiates a

series of cellular events leading to the destabilisation of the cell membrane and release of inflammatory mediators. This results in mucosal inflammation, wheezing, coughing, sneezing and nasal blockage.

Atopy can be diagnosed by (i) a positive skin prick test in
5 response to a common allergen; (ii) detecting the presence of specific serum IgE for allergen; or (iii) by detecting elevation of total serum IgE.

Genetic factors underlying a disease may be identified through localisation to particular chromosomal regions by genetic linkage. Genetic linkage is established by the study of families. It relies on matching the
10 inheritance of disease with genetic polymorphisms of known localisation (known as "genetic markers"). In a complex disease such as asthma, genetic linkage will typically localise genes to within 10 - 20 Megabases (Mb) of DNA. A region of this size may contain 350 - 700 genes, and will be too large to permit immediate identification of the disease-causing gene.

15 Closer localisation of disease-causing genes may be accomplished by the detection of associations between particular alleles and the disease phenotype. Over short segments of DNA, distinctive alleles of the individual polymorphisms will show non-random association with alleles of neighbouring polymorphisms. This phenomenon, known as "linkage
20 disequilibrium" occurs over 50-500 Kilobases (Kb) of DNA. Linkage disequilibrium may be detected by the study of individuals as well as by the study of families.

Disease-causing alleles will be in linkage disequilibrium with non-functional polymorphisms from the same chromosomal segment. It is
25 therefore possible to detect allelic association with disease from particular chromosomal segments, without identifying the exact polymorphism and gene underlying the disease state.

The detection of allelic association may therefore give information as to disease susceptibility in a particular individual. Furthermore, allelic association is indicative of a disease-causing gene being present within 500 Kb of DNA in either direction from the allele (i.e. 1 Mb in total). Such a
5 region may contain only 30 genes, within which the identification of the disease-causing gene is possible.

The presence of linkage disequilibrium also means that other polymorphisms may be anticipated to associate with disease, and that these additional polymorphisms will also be diagnostic of disease susceptibility in
10 particular individuals.

Genetic associations with atopy have been demonstrated. WO 95/05481 discloses that variants of the gene encoding the β -subunit of the high-affinity receptor for IgE ($Fc\epsilon RI\beta$) are associated with atopy. It teaches a method for diagnosing atopy which is based upon the
15 demonstration of the presence or absence of one of two variants in a specific portion of the DNA sequence of the gene encoding $Fc\epsilon RI\beta$, located near the commencement of exon 6 of the $Fc\epsilon RI\beta$ gene on chromosome 11. A further variant has also been found in which the unusual variant sequence is in the coding sequence for the C-terminal cytoplasmic tail of $Fc\epsilon RI\beta$ (4).

20 Tumour Necrosis Factor (TNF) is a pro-inflammatory cytokine that is found in increased concentration in asthmatic airways (5). We have previously shown that polymorphisms within the TNF gene are associated with an increased risk of asthma (6).

The known polymorphisms do not account for all of the genetic
25 factors which predispose to atopy. Identification of further genetic polymorphisms linked to atopy will allow the identification of individuals with susceptibility to atopy, for example children at risk before atopic disease has developed, with the potential for prevention of disease. The presence of

particular polymorphisms may predict the clinical course of disease (e.g. severe as opposed to mild) or the response to particular treatments. This diagnostic information will be of use to the healthcare, pharmaceutical and insurance industries.

5 We have previously established linkage of atopy to chromosome 13 (8). However, this finding is of no use in diagnosis.

It has now been discovered that a genetic polymorphism known as D13S273*4 on chromosome 13 is associated with atopy and can be used as a diagnostic tool.

10 The invention therefore provides a method for diagnosing an individual as being atopic, or as having a predisposition to atopy, which method comprises demonstrating in the individual the presence or absence of an allele which is associated with atopy, wherein the allele is situated at a locus in a region of chromosome 13 of up to 1 megabase in length, which
15 region contains the locus D13S273.

The 1 Mb region of chromosome 13 in which the D13S273 locus is situated flanks the D13S273 locus. Thus, the specific allele D13S273*4, or other polymorphisms in the region which are associated with atopy, may be the subject of identification in the method according to the invention. Equally,
20 two or more such alleles may be the subject of identification.

Current diagnostic methods involving detection at the nucleic acid level normally comprise the steps of:

- (i) obtaining a suitable tissue sample from the individual;
- (ii) preparing from the tissue sample a nucleic acid sample;
- 25 (iii) analysing the nucleic acid sample for the presence or absence of the relevant nucleic acid sequence, such as a specific allele.

Preferably, an amplification step is performed prior to the analysis, such that the locus at which the allele is situated is amplified. A preferred amplification technique is the PCR, although any suitable method of nucleic acid amplification may be employed.

5 In further aspects, the invention provides a pair of oligonucleotide primers for amplification of an allele which is associated with asthma, which allele is situated at a locus in a region of chromosome 13 of up to 1 megabase in length, which region contains the locus D13S273; and an assay kit comprising the pair of oligonucleotide primers.

10 The specific allele for identification may take the form of microsatellite repeats, which are nucleotide sequences containing short, repeated nucleotide motifs, usually a dinucleotide or a trinucleotide motif. A pair of primers which hybridize under suitably stringent conditions, to sequences at a position on either side of the microsatellite repeats, may be
15 used to amplify the microsatellite repeats by PCR. Differences in the number of repeats are recognised by size differences in the PCR products. An allele which has a specified number of repeats and therefore a known size can thus be identified. D13S273*4 is one such allele.

The primers employed in the method comprise nucleic acid
20 sequences which are complementary to, or substantially complementary to unique sequences either side of the microsatellite repeats, such that only the relevant polymorphic region of the genome is amplified. The conditions under which the amplification is performed are gauged such that specific hybridization of the primers to the flanking sequences occurs and non-specific
25 hybridization is avoided. The hybridization conditions are suitably stringent for that purpose. Standard techniques can be used to identify an appropriate set of reaction conditions.

Typically, the PCR products are detected by means of a detectable label attached to one of the PCR primers. Alternatively another form of labeling may be used such as a labeled sequence specific probe which hybridizes to the amplified sequences. The label may be a fluorescent or other label. The PCR products are subjected to size determination, typically involving size-separation for example by gel electrophoresis, and the presence or absence of the allele of interest is determined.

It will be evident that the invention is not limited with regard to the manner in which the presence or absence of the allele of interest is determined. The labeling, detection, separation or any other aspect of the method as described here may be replaced by other suitable known techniques and reagents.

The allele for identification may be an allele other than D13S273*4 which is in linkage disequilibrium with D13S273*4 and is associated with asthma. This includes alleles of both functional and non-functional polymorphisms. Functional polymorphisms include polymorphisms within genes, usually within coding sequences of genes. Non-functional polymorphisms are polymorphisms which do not themselves cause the disease.

This invention will now be further described in the Examples section which follows. The Examples are intended to be illustrative and do not limit the scope of the invention in any way.

EXAMPLES

25 Description of Laboratory Testing

Subjects:

Two panels of subjects have been studied.

Panel A consisted of 80 nuclear families sub-selected from an Australian population sample of 230 families (8). The panel contained a total of 203 offspring forming 172 sib-pairs. Fifty-two % of the children were atopic.

Panel B consisted of 77 nuclear and extended families recruited
5 from atopy and allergy clinics in the United Kingdom. These families contained 215 offspring forming 268 sib-pairs. Sixty-one % of the children were atopic.

Phenotypes

10 Skin tests to House Dust Mite (HDM) and mixed grass pollen (less the response of negative controls), specific IgE titres to HDM and Timothy Grass, and the total serum IgE were measured. A "Skin Test Index (STI)" was calculated as the sum of the prick skin test results to HDM and grass mix. "Atopy" was defined as a STI > 5mm, or a RAST score to HDM
15 and Timothy Grass > 2, or a total serum IgE > the 7th decile of the age-corrected population.

Genotypes

The microsatellite markers were typed by semi-automated
20 fluorescent methods, as described previously (8).

The polymerase chain reaction primer sequences for the marker D13S273 were as follows:

D13S273 5' CTG NGG CAA AAA CAA CTC TT (SEQ ID NO: 1)

UD13S273 5' ATC TGT ATG TCC TCC TTT CAA TG (SEQ ID NO: 2)

25 The polymerase chain reaction conditions were as follows:

The reaction volumes were 10µl, containing 50ng of genomic DNA, 200mM dNTPs, 1 x NH4+ buffer, 50ng oligonucleotide primers (forward labelled fluorescently), 0.5 to 3.0mM MgCl₂ and 0.2U Taq polymerase. Cycling

conditions were 1 min at 95°C, 1min at 55°C and 45s at 72°C; 28 cycles were used. PCRs were performed on an Hybaid Omnigene thermal cycler.

Electrophoresis and allele scoring were as follows:

- 5 PCR products were mixed with a size standard (GS350 TAM) in loading buffer (80% (v/v) formamide, 20% (v/v) 50mM EDTA, 0.1% (w/v) blue dextran). Samples were denatured at 95°C for 4min immediately prior to loading onto a 6% polyacrylamide gel and were electrophoresed at 800v for 6h on an Applied Biosystems (ABI) 373 DNA sequencer. Allele sizes were assigned using the
10 ABI GENESCAN and ABI GENOTYPER software.

Association Analysis

Association was tested against the phenotype of atopy by the Transmission Disequilibrium Test.

15

Results

- Association with the atopy phenotype was seen in each panel for allele 4 of D13S273 (D13S273*4). This allele is 238 base pairs in size, using the primers described above. (Other primers can be designed and their
20 amplification product size determined for D13S273*4, using known sequence information (9).) The results of TDT testing were as follows:

	Panel A			Panel B			combined		
	T	N	p	T	N	p	T	N	p
Maternal	22	24	.0081	36	18	.0099	58	26	.00048
Paternal	13	24	ns	25	28	ns	38	52	ns

The results indicate that D13S273*4 shows a strong reproducible association with atopy in two diverse panels of subjects. It may therefore be inferred that a gene influencing atopy is present within 500 kilobases in either direction of D13S273.

5

REFERENCES

1. Strachan, D.P., Anderson, H.R., Limb, E.S., O'Neill, A. and Wells, N. (1994). A national survey of asthma prevalence, severity, and treatment in Great Britain. *Arch. Dis. Child.*, **70**, 174-178.
- 10 2. Fraser, R.S., Paré, J.A.P., Fraser, R.G. and Paré, P.D., eds. (1994) Synopsis of Diseases of the Chest. WB Saunders Company, Philadelphia: 635-53.
3. Djukanovic, R., Roche, W.R., Wilson, J.W., Beasley, C.R., Twentyman, O.P., Howarth, R.H. and Holgate S.T. (1990) Mucosal inflammation in
15 asthma. *Am. Rev. Respir. Dis.*, **142**, 434-457.
4. Hill, M.R. and Cookson, W.O.C.M. (1996). A new variant of the β subunit of the high-affinity receptor for Immunoglobulin E (Fc ϵ RI- β E237G): Associations of measures of atopy and bronchial hyper-responsiveness. *Hum. Mol. Gen.* **5**, 959-962.
- 20 5. Broide, D.H., Lotz, M., Cuomo, A.J., Coburn, D.A., Federman, E.C. and Wasserman, S.I. (1992) Cytokines in symptomatic asthma airways. *J. Allergy Clin. Immunol.* **89**, 958-967.
6. Moffatt M.P, Cookson WOCM. Tumour Necrosis Factor Haplotypes. and Asthma. *Hum Molec Genet* 1997; **6**: 551-554.
- 25 7. Bailly S, di Giovine FS, Blakemore A.I.F, Duff G.W. Genetic polymorphism of human interleukin-1 α . *Eur J Immunol* 1993; **23**: 1240-1245.
8. Daniels S.E, Bhattacharyya S, James A, Leaves N.I, Young A, Hill M.R, Faux J.A, Ryan G.F, le Souef P.N, Lathrop G.M, Musk A.W, Cookson

9. Dib S. et al. A comprehensive map of the human genome based on 5264 microsatellites. *Nature* 1996; **380**: 152-154.

10. Jorde L, Watkins W.S, Carlson M, Groden J, Albertsen H, Thliveris A, Leppert M.E. Linkage Disequilibrium in the adenomatous polyposis coli region. Am J Hum Genet 1995; 54: 884-98.